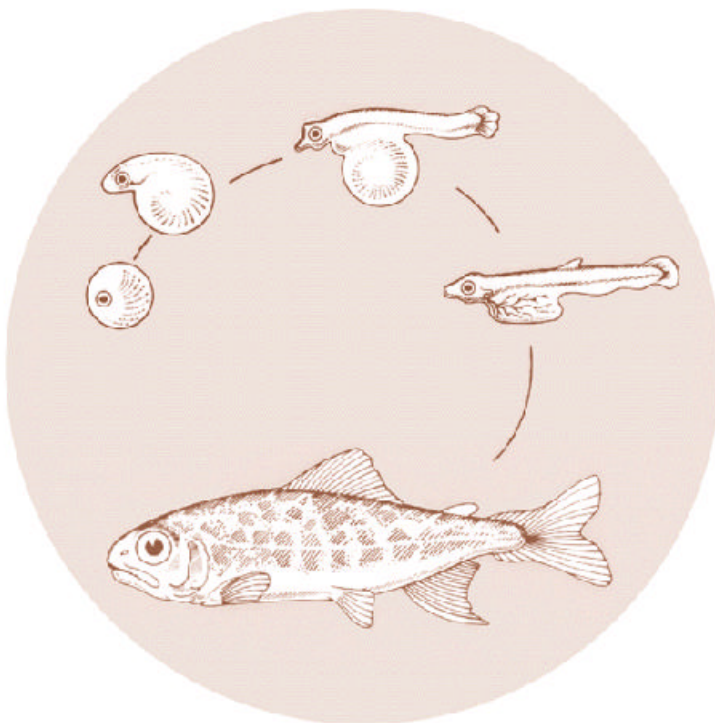# EVALUATION OF A SUBUNIT VACCINE TO INFECTIOUS HEMATOPOIETIC NECROSIS (IHN) VIRUS

Annual Report 1984

DOE/BP-16479-1

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FOREWORD

The Bonneville Power Administration (BPA) strongly encourages publication of Its project reports in peer-reviewed scientific journals. To promote and assist this goal, project leaders may, upon often approval from BPA, substitute a manuscript for publication in lieu of an annual report. Therefore the represents both a manuscript for publication and an annual report. However, figures 4 and 5 are confidential and omitted here because they contain information which is the subject of a patent application.

Evaluation of a Subunit Vaccine to Infectious

Hematopoietic Necrosis (IHN) Virus

Annual Report FY 1984

by

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Prepared for

ABSTRACT

A prototype subunit vaccine to IHN virus is being developed by recombinant DNA techniques. The techniques involve the isolation and characterization of the glycoprotein gene, which encodes the viral protein responsible for inducing a protective immune response in fish. The viral glycoprotein gene has been cloned and a restriction map of the cloned gene has been prepared.

Preliminary DNA sequence analysis of the cloned gene has been initiated so that manipulation of the gene for maximum expression in appropriate plasmid vectors is possible. A recombinant plasmid containing the viral gene inserted in the proper orientation adjacent to a very strong lambda promoter and ribosome binding site has been constructed. Evaluation of this recombinant plasmid for gene expression is being conducted. Immunization trials with purified viral glycoprotein indicate that fish are protected against lethal doses of IHNV after immersion and intraperitoneal methods of immunization. In addition, cross protection immunization trials indicate that Type 2 and Type 1 IHN virus produce glycoproteins that are cross-protective.

Infectious hematopoietic necrosis virus is a major pathogen in Columbia River steelhead and salmon fish stocks. The incidence of IHNV infections in hatcheries along the Columbia River has risen at an alarming rate. More than 25 million eggs and fish have been destroyed in the past four years in attempts to control the disease. The potential value of this fisheries resource has been estimated at over $230 million (Figure 1). Thus, the goal of this project under Program Measure 704 (h) (4) is to develop and test an effective, safe, and economical vaccine for IHNV.

Control of IHNV

Presently, the only practical method available to control the spread of IHNV is the removal and destruction of all infected fish, the disinfection of all ponds and equipment, and the restocking of the hatchery with virus-free eggs. Since IHNV is believed to be transmitted with eggs as an external contaminant, eggs are disinfected with iodophore treatment for ten minutes at pH 6.0. The effectiveness of this treatment is still controversial since eggs are water-hardened before the iodophore bath. The water hardening process may allow virus to enter the egg and thus make the virus insenstive to iodophore treatment (Groberg and Fryer, 1983).

POTENTIAL VALUE OF FISH DESTROYED BECAUSE OF IHNV - 1980-84	
23,326,000+	Fish and Eggs*
506,520	2% Return
$230,466,600+	$455/fish**

*W. Groberg, personal communication
**Meyer, 1982. Estimated average for
 Columbia River Chinook Salmon and
 Steelhead trout.

Another method of control of the disease is the rearing of susceptible fish at water temperatures above 15 ° C. This method is only feasible on a large scale where 15 ° C. water temperatures are easily and economically obtained. This method has been used successfully to control IHN virus disease in chinook salmon in the Sacramento River (Amend, 1974).

An attenuated strain of IHN virus has been developed (McMichael, 1974). It was produced by transferring the Oregon sockeye salmon virus isolate on steelhead trout cells in tissue culture for more than forty passages. The attenuated virus did immunize sockeye salmon fry or juveniles after contact with the virus for 48 hours. In most trials, 90% or more of the vaccinated fish were protected against a fatal infection with the wild-type virus for as long as 110 days (Tebbit, 1976; Fryer et al., 1976). However, this strain is virulent in rainbow trout and the reversion frequency from nonpathogenic to pathogenic wild-type virus was not determined (Fryer, personal communication).

Thus, substantial effort has been directed toward the development of a subunit viral vaccine for IHN virus. The parameters governing the efficacy of any subunit viral vaccine for IHN virus have not been determined. It is clear that the following questions must be asked in the evaluation of any IHNV vaccine:

1. Since IHNV kills very young fish, what is the earliest stage in the developing fish when protective immunity is inducible?

2. How long does protective immunity last?

3. Will vertical transmission of IHNV be interrupted if brood stock fish are immunized?

4. Will the vaccine prepared against one strain of IHNV induce protection against another strain of IHNV?

5. Are all salmonid species equally responsive to the vaccine?

6. What methods of preparing the vaccine will enhance the potency of the vaccine?

These are just some of the questions that are being asked by the research undertaken in this contract.

THE VIRION GLYCOPROTEIN OF INFECTIOUS HEMAPOIETIC

NECROSIS VIRUS INDUCES PROTECTIVE IMMUNITY

IN SALMONID FRY

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INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus that infects salmon and trout. It introduces an acute disease resulting from the destruction of the hematopoietic tissue in the kidneys and may lead to the loss of an entire fish hatchery population (ref.). At the present time the only effective means for controlling this disease is the complete destruction of stocks of infected fish and sterilization of hatchery. These control methods are expensive and in some cases lead to the destruction of valuable fish stocks. Thus, an effort was made to develop a vaccine for IHNV.

Previous studies have indicated that protective immunity to IHNV was induced in sockeye salmon (Oncorhynchus nerka, Walbaum) with a strain of IHNV which had been passed on steelhead trout cells in tissue culture for more than 40 times (Tebbit, 1976; Fryer et al., 1976). However, the attenuated strain was not effective as a vaccine in rainbow trout and was actually lethal in young trout (Winton, personal communication). In order to avoid some of the problems attendant with attenuated vaccines we are trying to develop a subunit vaccine to IHNV.

The identification of the viral protein responsible for the

induction of protective immunity in fish was undertaken. For the
rhabdoviruses, vesicular stomatitis virus(VSV) and rabies, the
viral glycoprotein is the only viral antigen which induces
neutralizing antibody and protective immunity. We expected that
IHNV would have a similar viral antigen. However, in the fish
system where the immune response is less clearly understood than
the mammalian system, it was necessary that the IHNV glycoprotein
be tested for its immunogenic properties. We report here that
purified IHNV glycoprotein will induce protective immunity in
salmonid fry. Immune induction occurs after intraperitoneal
inoculation or immersion in a solution of purified glycoprotein
and protective immunity is produced in fish as small as 0.5 g and
lasts for at least 30 days.

MATERIALS AND METHODS

Cells and Virus

The chinook salmon embryo cells, CHSE-214, were obtained from
J. L. Fryer, Oregon State University, Corvallis, Oregon and
the epithelioma papillosum cyprini cells (EPC) were obtained from
D. Mulcahy, National Fisheries Research Center, Seattle,
Washington. The cells were grown in Eagle's minimum essential
medium (MEM) supplemented with fetal calf serum (5 %), NaHCO$_3$
(0.075%), penicillin (100 i.u./ml) and streptomycin (100 ug/ml).
The CHSE-214 cells were used to prepare virus stocks and the EPC
cells were used for virus assays.

Assays were performed using confluent EPC cell monolayers grown in 96-well tissue culture plates (Linbro). Samples from infected fish were prepared as described (ref.), sterilized by filtration (0.2 um acrodisc, Gelman), and diluted in MEM (without fetal calf serum). Duplicate samples (0.05-0.1 ml) of each dilution were placed on monolayers in individual wells and allowed to adsorb for 60 minutes. Sample inoculum was removed from the wells after adsorption and l-l.5 ml of MEM growth medium was added to each well.

The Round Butte Type I strain of IHNV was obtained from W. Groberg, Oregon Department of Fish and Wildlife, and the Hagerman Valley Type 2 strain was obtained from N. Wood, Rangen Research Laboratories, Idaho. Purified viral glycoprotein was obtained from the Round Butte strain of IHNV after extensive purificiation of the virus by isopycnic and velocity sedimentation in an ultracentrifuge. All virus strains were prepared by growing the virus at a multiplicity of infection of 0.01 to 0.001 TCID5O per cell on CHSE-214 cells as previously described (ref.).

The virus used for challenge doses in the immunization trials was prepared from a stock of virus which had undergone no more than three passes in tissue culture after isolation from infected fish.

Purification of the Viral Glycoprotein

Purified IHN virus (2 mg) was prepared as recently described (Kurath and Leong, 1985) from 2 liters of the supernatant fluid from infected cells. The virus was suspended in 2 ml of 0.01 M Tris-hydrochloride, pH 7.6. Briton X-100 (Eastman Kodak, scintillation grade) was added to the suspension to a final concentration of 1%. The mixture was centrifuged in a Beckman SW 50.1 rotor at 45,000 rpmfor 60 min at 4°C. The supernatant fluid containing purified glycoprotein was analyzed by polyacrylamide 1.5 gel electrophoresis and found to free of other containing viral proteins (Figure 1). The purified glycoprotein was stored at -70° C in 0.01 M Tris-hydrochloride, pH 7.6. Approximately 50% of the viral glycoprotein was recovered. The usual yield was 200-300 ug at 40-50 ng/ul.

Because Triton X-100 is toxic to fish at concentrations above 0.01%, the detergent had to be removed from the glycoprotein solution before it was used to immunize fish. The detergent concentration was reduced to less than 0.01% after five batch elutions through SM-2 Biobeads (Bio Rad). Protein recoveries after batch elution ranged from 50-90% The final glycoprotein concentration was determined by estimation on silver-stained polyacrylamide gels with known concentration markers.

<u>Analysis of purified glycoprotein by SDS-polyacrylamide gel electrophoresis (PAGE).</u>

Electrophoresis was performed in a 10% polyacrylamide gel with a 5% stacker as described by Laemmli (1970). Peptide bands were visualized by staining the gel with silver nitrate as described by Allen (1980).

<u>Immunization of fish</u>

The fish utilized in these studies were obtained from Oregon Department of Fish and Wildlife (ODFW) hatcheries through Richard Holt, Oregon State fish pathologist. The fry were obtained one week before immunization and acclimated at the Fish Disease Laboratory in Corvallis, Oregon. The fry were maintained on BioDiet (BioProducts, Warrenton, Oregon) in well water flowing at 0.5 gallons per minute at 12°. When the fish were 0.5 g in size they were vaccinated by direct immersion or intraperitoneal inoculation.

The fish were anesthetized with benzocaine and injected intraperitoneally with 10 ul of purified IHNV glycoprotein from a 2% ul glass syringe with a 30 gauge disposable needle. The inoculated fish received approximately 450 ng of purified glycoprotein.

Immersion immunization was performed on non-anesthetized fish in a beaker containing purified glycoprotein (40-50 ug/ml) in

0.01 M Tris-hydrochloride, pH 7.6. The fish were exposed to the protein solution for 1-2 minutes and then released into a holding tank containing running water at 12°.

All immunized and mock immunized fish were maintained in well water flowing at 0.5 gallons per minute at 12 o for 30-60 days before challenge with live IHN virus.

Virus challenges.

Challenges were made with duplicate lots of 25 vaccinated and unvaccinated control fish for each virus dilution. The fish were exposed to different virus dilutions in 1 liter of water containing 10-2, 10^{-3}, 10^{-4}, or 10^{-5} dilutions of the virus stock. These dilutions represented approximately 2 X 10^{6}, 2 X 10^{5}, 2 x 10^{4}, and 2 X 10^{3} TCIDSO doses per ml of water. The fish were held in the virus-containing water for 18 hours and then returned to holding tanks. Dead fish were removed daily, recorded, weighed, and processed for IHN virus isolation.

Isolation of IHNV from infected fish.

Dead fish were processed immediately for virus isolation. The fish were weighed and diluted (w/v) 1:10 with Hanks Buffered Salt Solution, and then macerated in a stomacher processor (Tekmar). The resulting suspension was clarified by centrifugation and the supernatant solution was treated with antibiotics overnight at 4 °. The next day the fluid was

incoulated directly onto CHSE-214 cells and EPC cells in multi-well plates as previously described (Engelking and Leong, 1970). The cells were observed daily for cytopathic effects for two weeks. For those samples where CPE was questionable, the tissue culture fluid from the sample well was removed and reinoculated onto CHSE-214 cells and subsequently labeled with ^{35}S methionine as described (Hsu et al., 1985).

Only those fish from which IHNV was isolated were considered in these studies. The percentage mortality was determined for each group and the relative percentage survival was calculated for each group of vaccinated compared to controls as follows:

Relative percentage

Survival = (1 - % specific loss vaccinated) X 100

% specific loss controls

RESULTS

Purification of the Viral Glycoprotein.

In order to prepare the IHN virus glycoprotein in a biologically active form, purified IHN virus was treated with Triton X-100 at protein:detergent ratios of 1:25, 1:100, 1:200, 1:400, 1:600, and 1:1000. At a protein:detergent ratio of 1:100, approximately 50% of the viral glycoprotein was released from the virus. Subsequent analysis of the purified glycoprotein obtained after treatment of the virus with 1% Triton X-100

(protein:detergent ratio of 1:100) indicated that the preparation
was free of any contaminating virion proteins such as M-l and M-2
(Figure 1). Although viral glycoprotein was released at higher
protein:detergent ratios without other contaminating virion
proteins, the higher detergent concentrations were not used
because it was not possible to reduce the Triton X-100
concentratin to 0.01%. Detergent concentrations greater than 0.01%
were toxic to the fish (Table 1).

Immunization of Fish with Purified IHNV Glycoprotein.

     Four experiments; were carried out with different stocks of
kokanee fry   Ocorhynchus nerka) and one experiment was performed
on rainbow trout fry (Salmo gairdneri). The fish were immunized
by inoculation of the purified glycoprotein and by immersion in a
solution containing the viral antigen. Because fish have been
shown to become refractory to the pathogenic effects of IHNV
infection after 6 months of age (ref.), the fish were immunized at
0.5 g body weight. After a period of 30-35 days, the immunized
and control non-immunized fish were challenged with ten-fold
dilutions of lethal, live IHNV as described in the Materials and
Methods.

     The data are presented as percent mortalities at each viral
dilution and provide an indication of the relative LD50 (Lethal
Dose for 50% mortality) for the control and immunized fish
(Figures 2-7). The data is summarized in Table 2 where the LD50
measurements for the control, immersed immunized, and inoculated

immunized groups are given.   Clear protection to IHNV infection was provided by immunization with purified glycoprotein as evidence by the higher LD50 for the immunized fish, i.e. 10-100 times more virus was required to kill 50% of the fish in the immunized group.

   The actual data are presented in Table 3 as relative percent survival for each experiment. In addition a plot of the cumulative mortality over time is shown in Figures 8 and 9. The analysis of this data indicates that The IHNV glycoprotein does induce protective immunity in young fish as small as 0.5 g for at least 50 days.   The inoculation route of vaccination was more effective than immersion vaccination.

Virus Challenge with a Different Virus Strain. One of the critical questions which must be answered in the development of any viral vaccine is that of cross immunity between different strains of the same virus.   Fish immunized with purified glycoprotein from the Round Butte strain of IHNV were challenged with a virus from the Hagerman Valley, Idaho, a Type 2 strain of IHNV (Hsu, 1984).   The first experiment of this type is presented in Figure 7 and Table 4.   The results suggest that Round Butte glycoprotein will induce a protective immunity against virus challenge with Hagerman Valley IHNV. Unfortunately, in some virus dilution groups there were non-specific mortalities and the numbers of fish included in the study were too small.

DISCUSSION

The envelope glycoprotein of IHNV has been purified and shown to induce protective immunity against challenge with live, virulent IHN virus. It appears that IHNV is similar to rabies and vesicular stomatitis virus, both mammalian rhabdoviruses with envelope glycoproteins that are solely responsible for inducing neutralizing antibody and protective immunity in the host (ref).

Protective immunity was induced by vaccination with 0.4-0.5 ng of purified IHNV glycoprotein. This quantity of viral protein is equivalent to an infectious dose of 2025 TCIDSO units of virus. The estimate is based on a calculation of 22% of the total virus protein as glycoprotein (Leong, Hsu, and Engelking, 1983) and a molecular weight of 7.46×10^7 daltons for the virus particle. The protein dose:body weight ratio used in these experiments was 0.5 ug/0.5 g or $1/10^6$, a dose approximately equivalent to the dose normally used to immunize mice and rabbits with purified rabies glycoprotein (Cox et al., 1980; Wiktor et al., 1973). The protein dose used in the rabies studies ranged from 12.5 ug of purified protein per adult mouse (30-35 g body weight) to 50 ug per adult rabbit (1135 g body weight). These comparisons suggest that the IHNV glycoprotein is highly immunogenic in fish.

Although these experiments were carried out for only one month the cumulative mortality data (Figures 8 and 9) suggest that the duration of immunity should last longer than that period of time. These studies did not include any experiments that accurately measure the duration of immunity because salmon and trout are refractory to the pathogenic effects of IHNV infection after 6 months (ref.). A method to detect immunization by other means than virus challenge must be developed for these studies. In addition, the effect of a booster dose on the duration of immunity must be determined.

The important observation is that the viral glycoprotein alone devoid of any other component of the virus particle is highly immunogenic. This seems to justify the earnest consideration of the production and application of such a preparation 2.3 a vaccine for fish.

Figure 1.   SDS-polyacrylamide slab gel electrophoresis of the purified glycoprotein of IHNV.   The glycoprotein was removed from purified IHN virus by treatment with 1% Triton X-100.   The supernatant fluid containing the purified glycoprotein was passed through SM-2 Biobeads and then analyzed by SDS-polyacrylamide gel electrophoresis.   Lane A.   High molecular weight markers: Myosin (200,000); Beta galactosidase (130,000); phosphorylase B (94,000); bovine serum albumin (68,000); and ovalbumin (43,000). Lane B. Low molecular weight markers: phosphorylase B (94,000); bovine serum albumin (68,000); ovalbumin (43,000); carbonic anydrase (30,000); soybean trypsin inhibitor (21,000); and lysozyme (14,300).   Lane C. Purified IHNV: L (150,000); G (67,000); N (40,000); M-l (21,000); and M-2 (17,500). Round Butte strain. Lane D.   Ten ul of purified IHNV glycoprotein. Round Butte strain. Lane E. Fiften ul of purified IHNV glycoprotein. Round Butte strain.   The protein bands were stained with silver nitrate.

Figure 2.   Determination of the LD50 for control, nonvaccinated fish and fish vaccinated by injection or immersion with purified IHNV glycoprotein -Round Butte strain.   The fish were challenged with different virus dilutions of live, virulent IHNV-Round Butte strain as described in Materials and Methods, The fish were kokanee fry obtained from Oak Springs Hatchery, Oregon. (Experiment 1)

Figure 3. Determination of the LD50 for control, nonvaccinated fish and fish vaccinated by injection or immersion as in Figure 2. The fish were rainbow trout fry obtained from Roaring River Hatchery, Oregon. (Experiment 2)

Figure 4. Determination of the LD50 for control, nonvaccinated fish and fish vaccinated by injection or immersion as in Figure 2. The fish were kokanee fry obtained from Wizard Falls Hatchery, Oregon.   (Experiment 3)

Figure 5. Determination of the LD50 for control, nonvaccinated fish and fish vaccinated by injection or immersion as in Figure 2. The fish were kokanee fry obtained from Wizard Falls Hatchery, Oregon. (Experiment 4)


Figure 6. Determination of the LD50 for control, nonvaccinated fish and fish vaccinated by injection or immersion as in Figure 2. The fish were kokanee fry obtained from Wizard Falls Hatchery, Oregon. (Experiment 5).

Figure 7. Cross immunization study. Determination of the LD50 for control, nonvaccinated fish and fish vaccinated by injection as in Figure 2. The fish were challenged with different dilutions of live, virulent IHNV-Hagerman Valley strain as described in the Materials and Methods. The fish were kokanee fry obtained from Wizard Falls Hatchery, Oregon.

Figure 8. Cumulative mortalities for control, nonvaccinated fish and fish vaccinated by injection or immersion. The fish are kokanee fry and the experimental data is for Experiment 1.

Figure 9. Cumulative mortalities for control, nonvaccinated fish and fish vaccinated by injection or immersion. the fish are rainbow trout fry and the experimental data is for Experiment 2.

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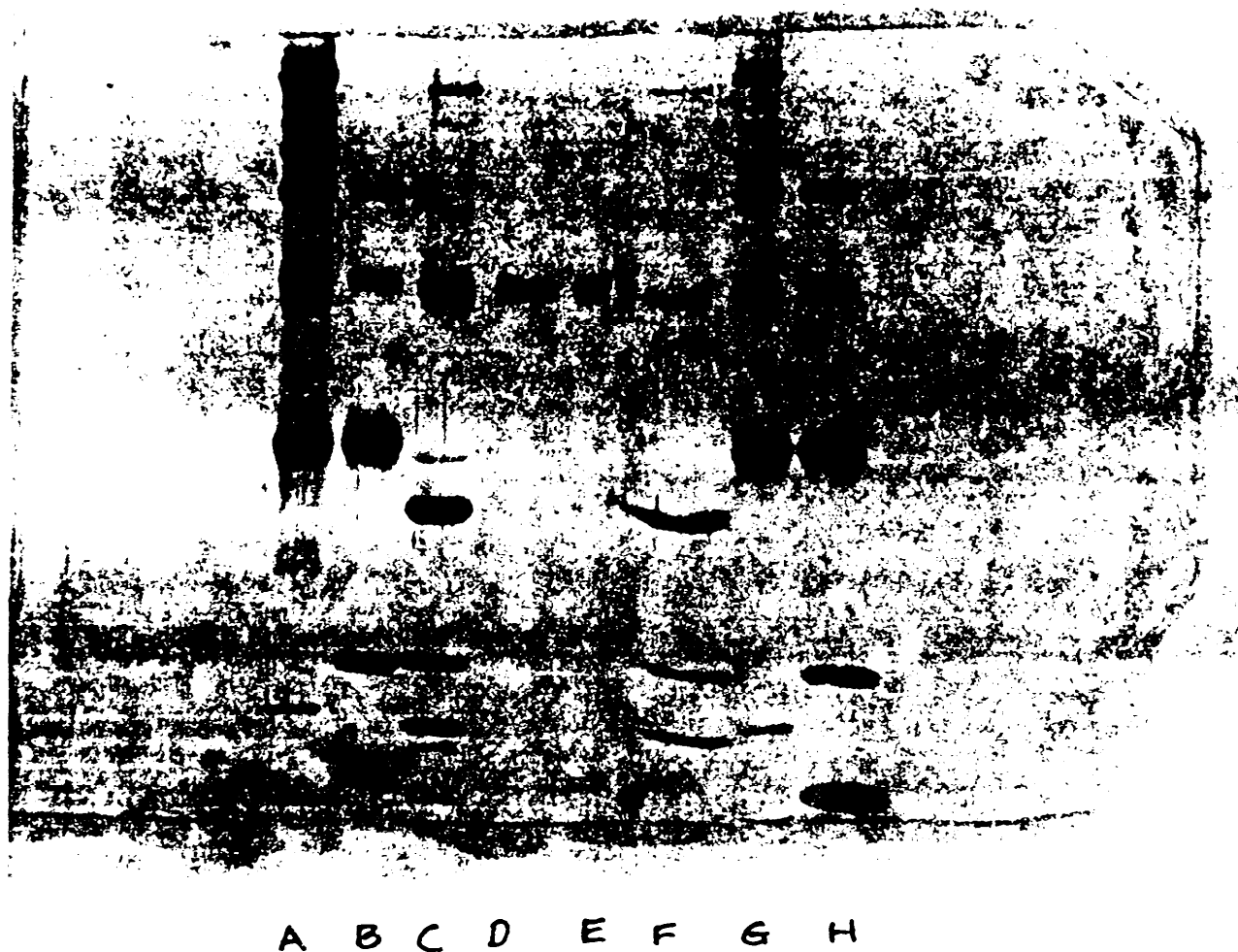
Silver strained gel of
purified G protein.
A= high molecular weight marker
B= low molecular weight marker
C= RB1 purified IHNV

D= 10 ul purified G protein
E= 15 ul purified G protein
F= purified IHNV, RB strain
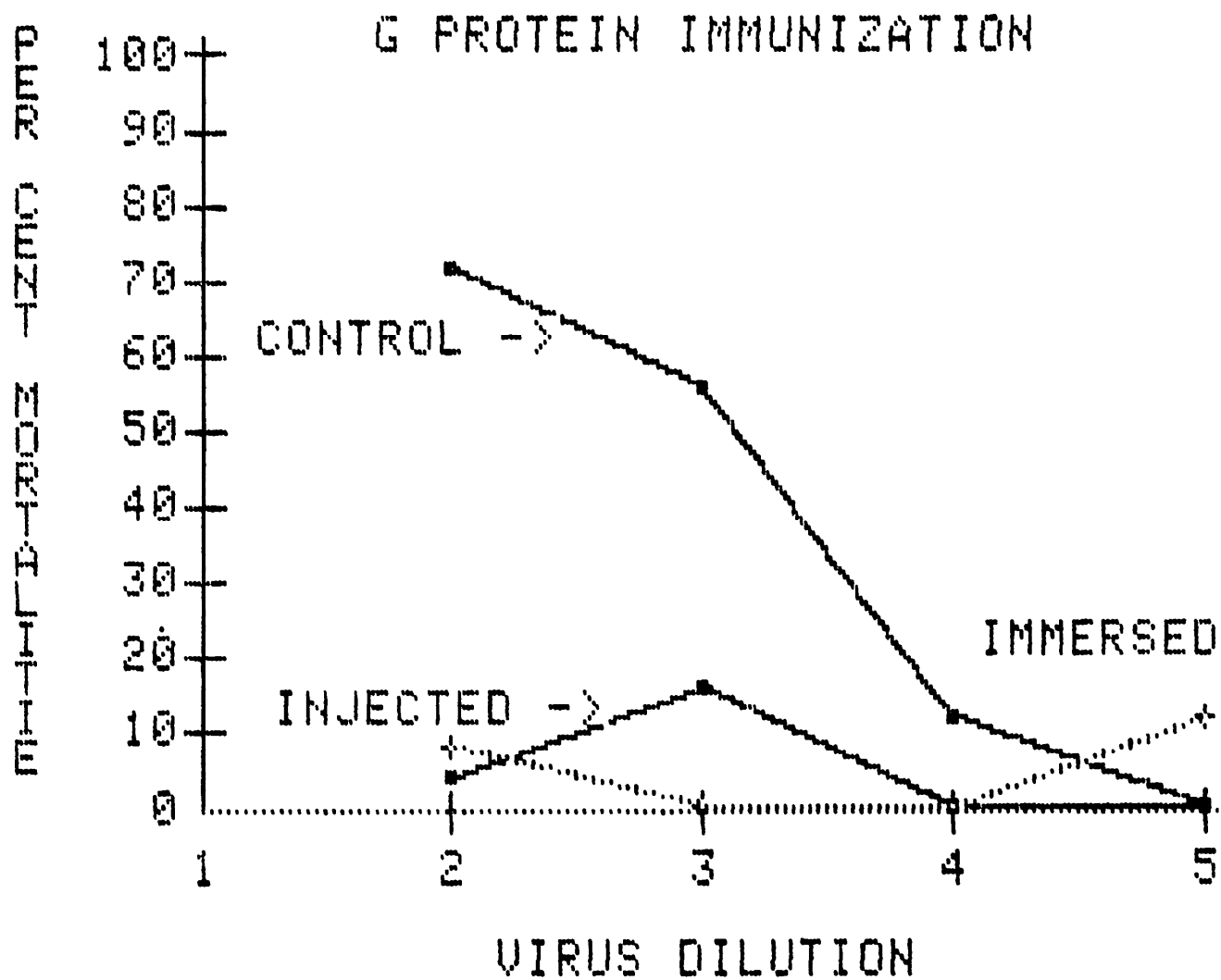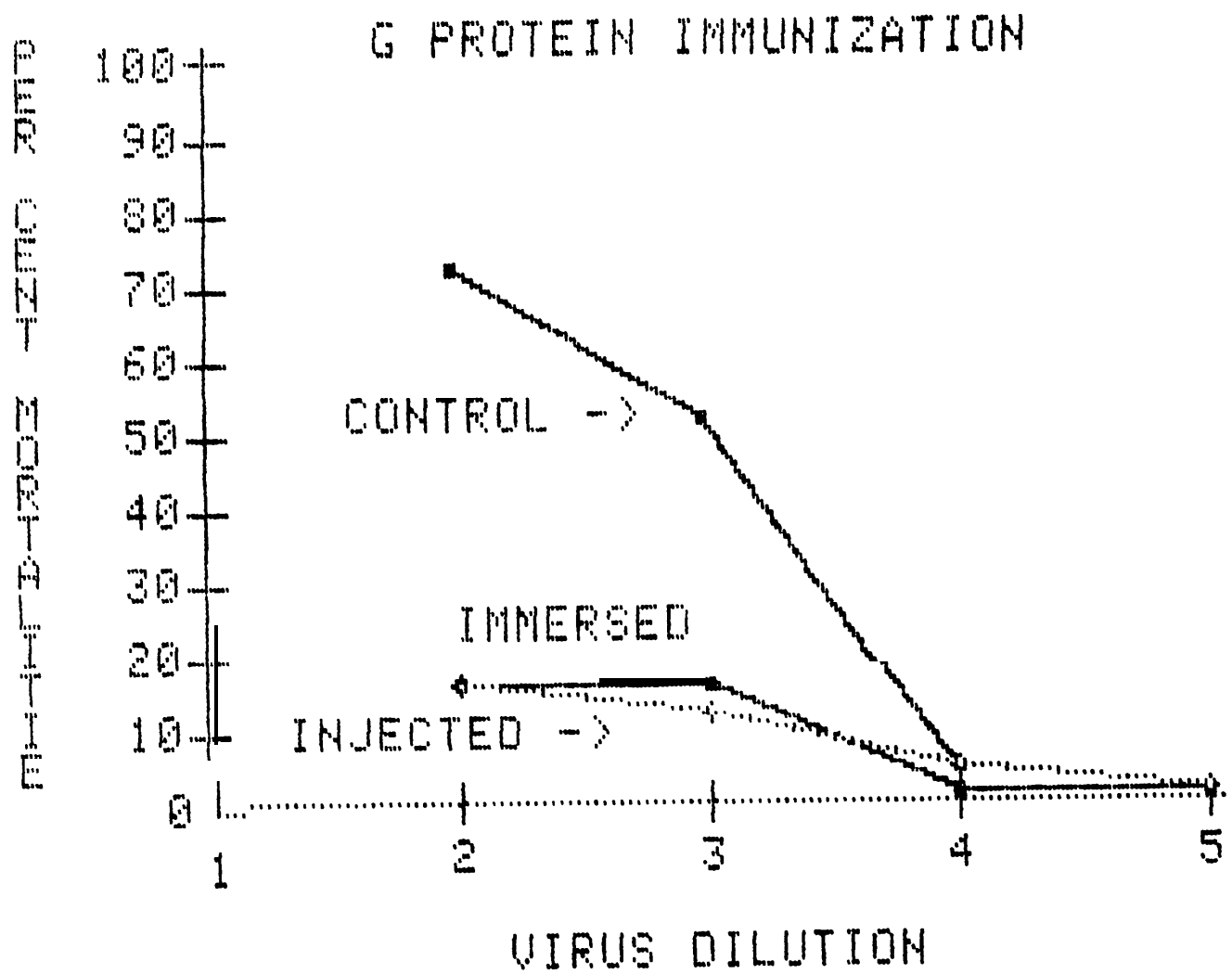G= high molecular weight marker
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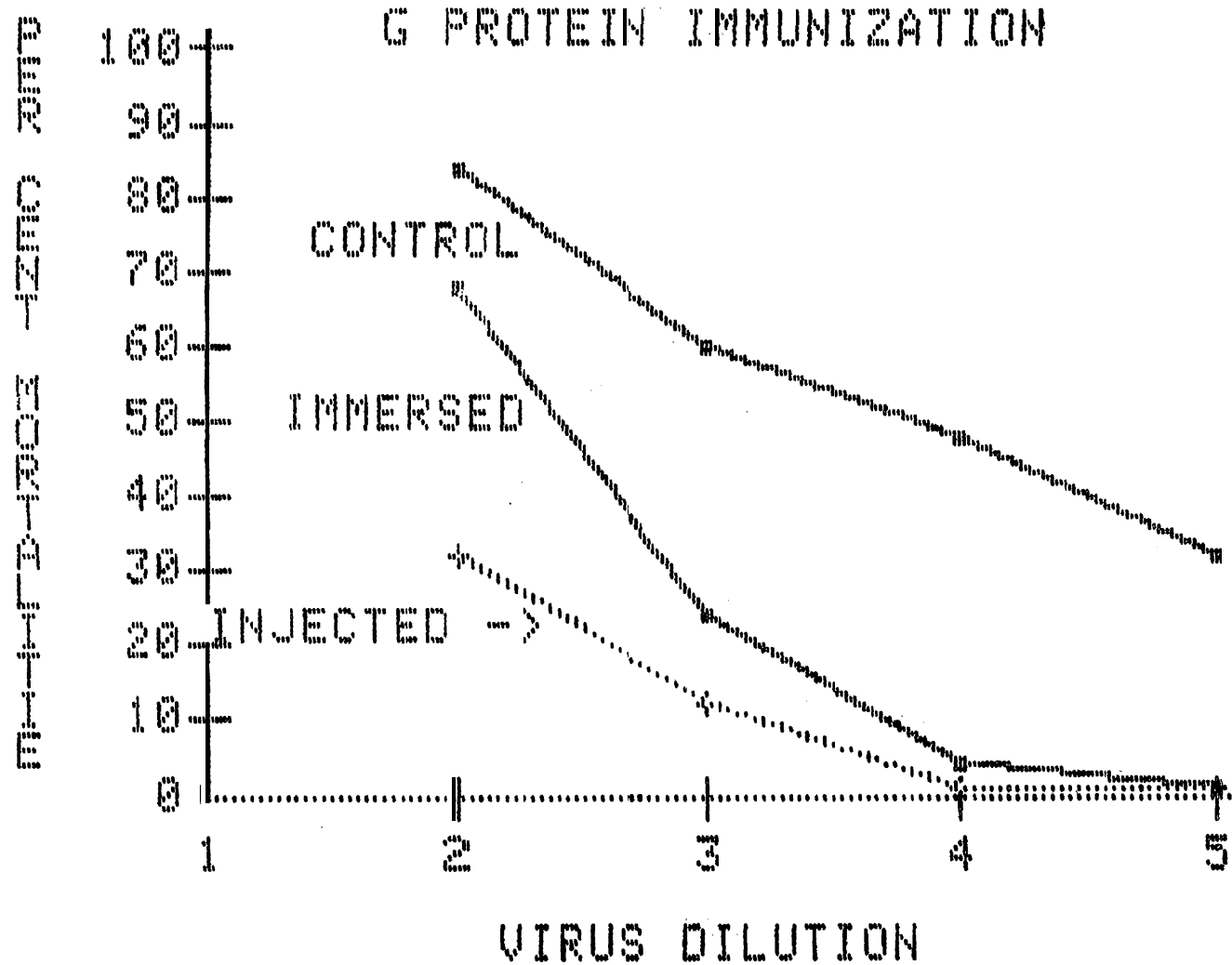
Figure 1

Figure 2

Figure 3

Figure

Figure 5

Figure 6

CROSS IMMUNIZATION OF IHNV
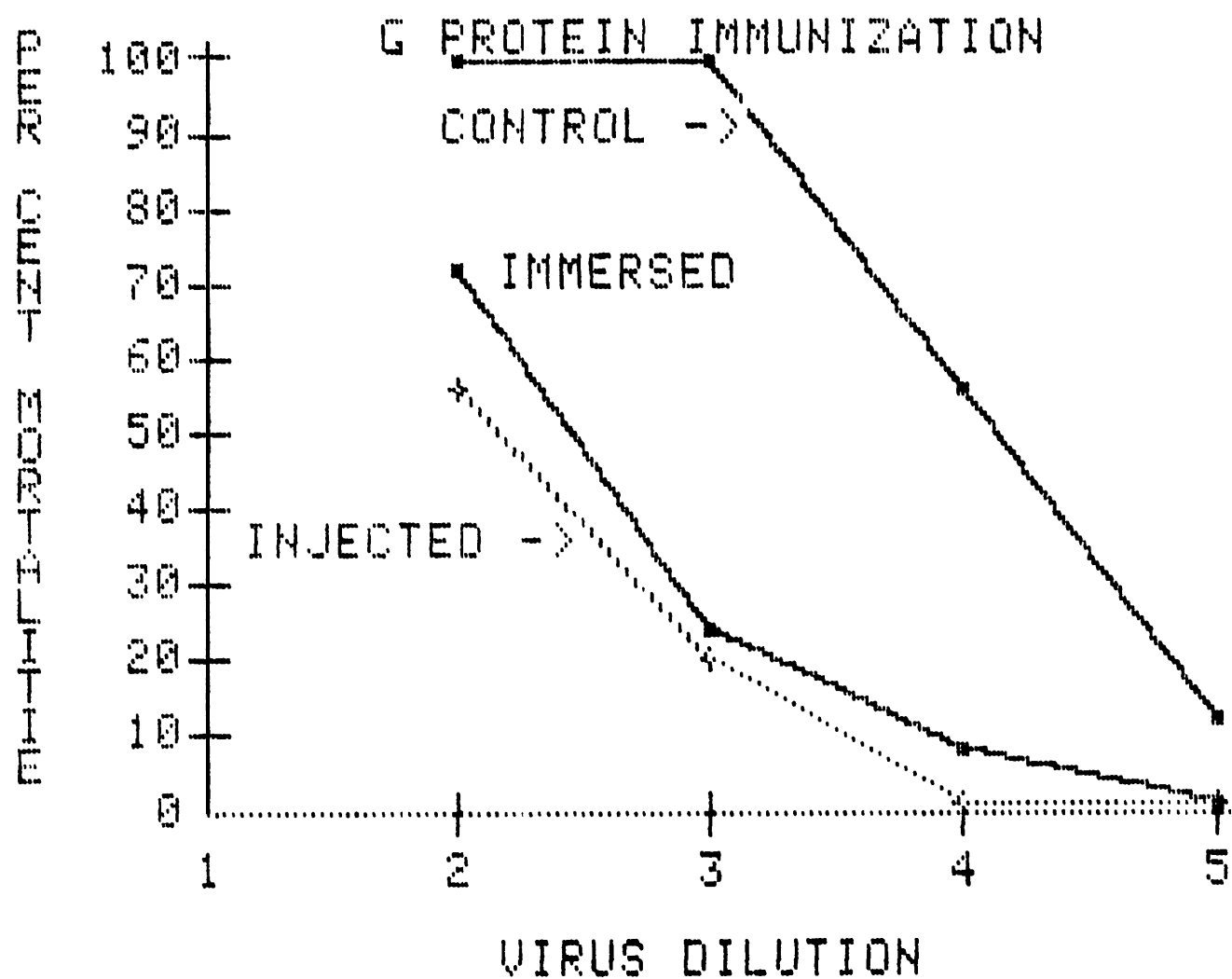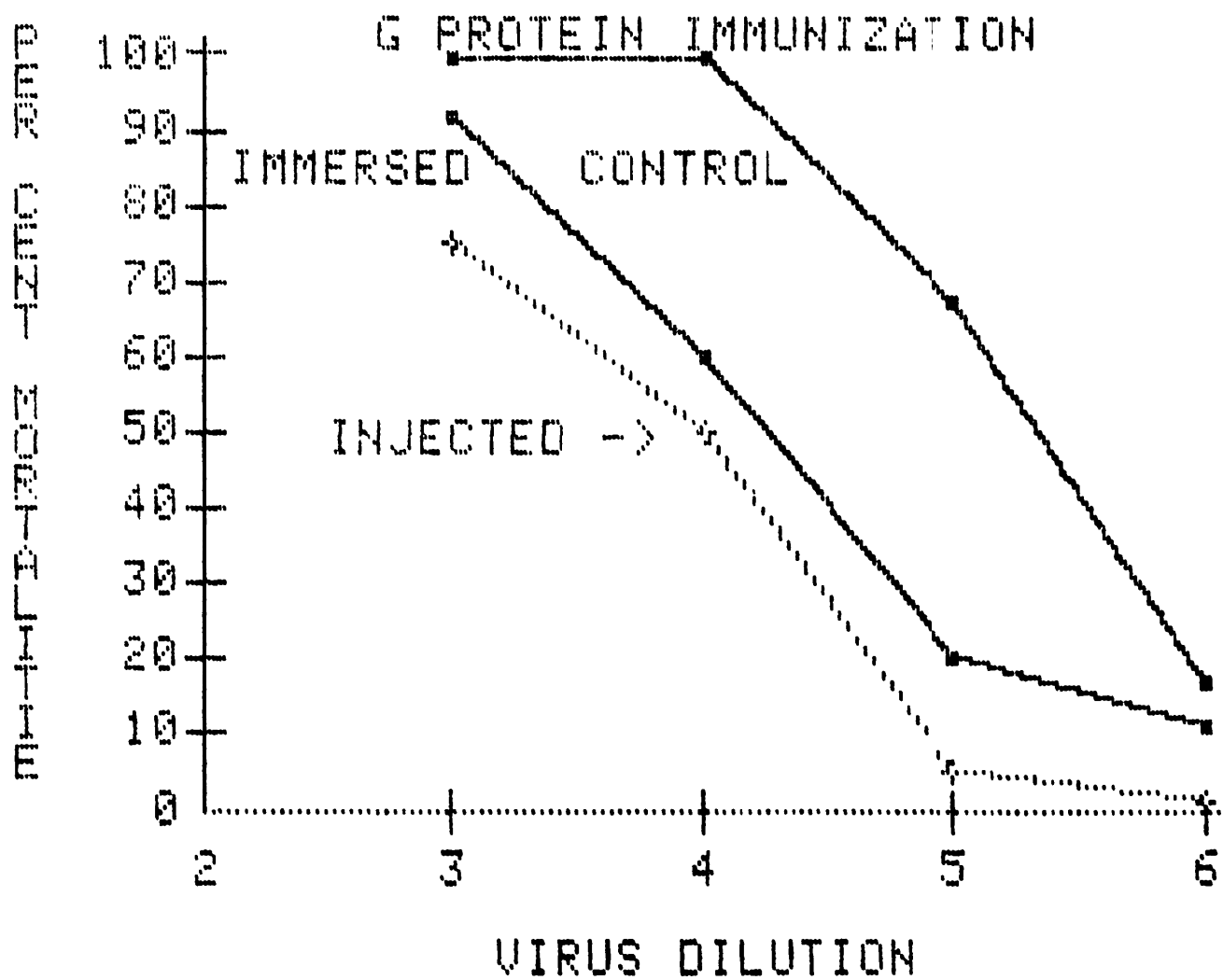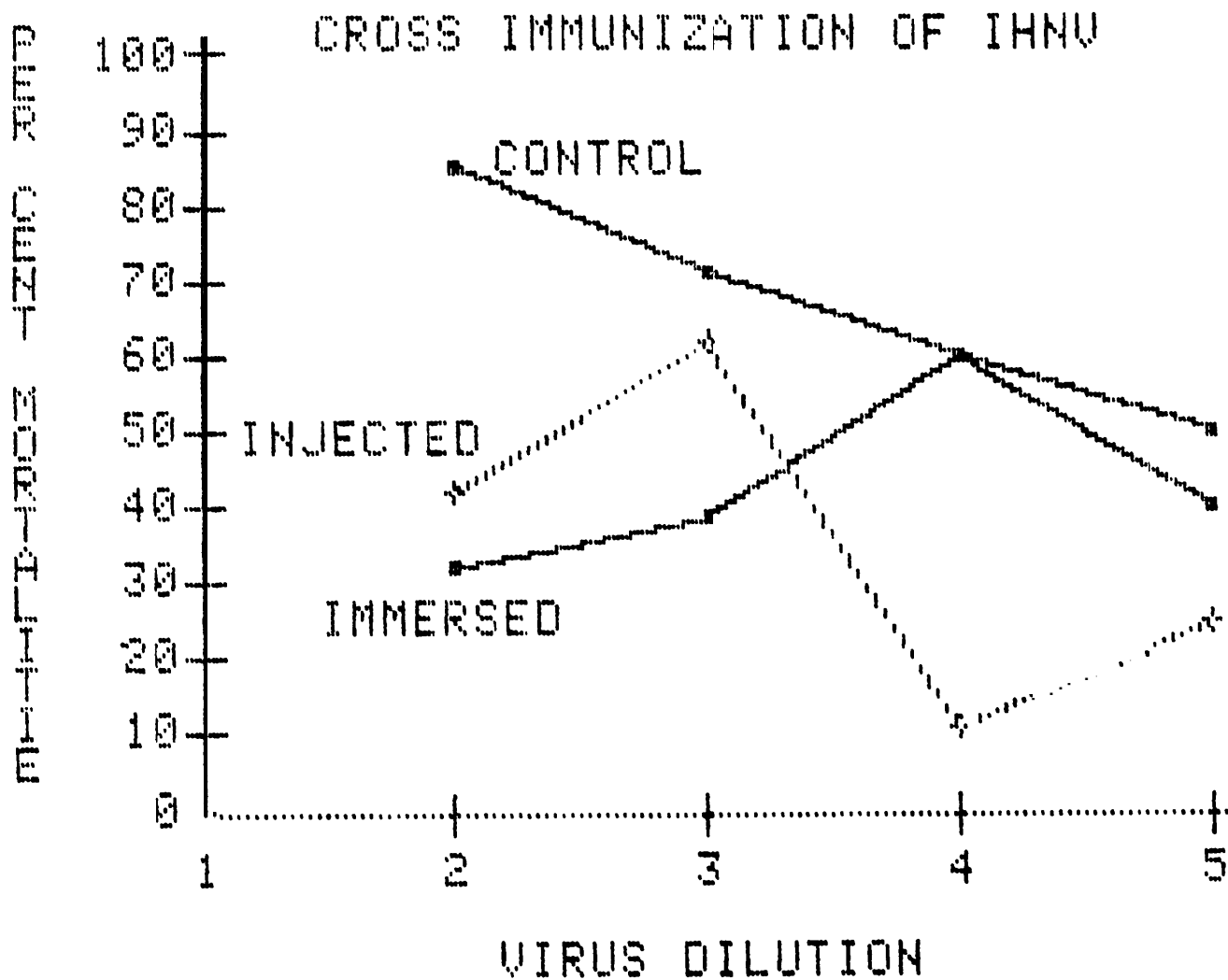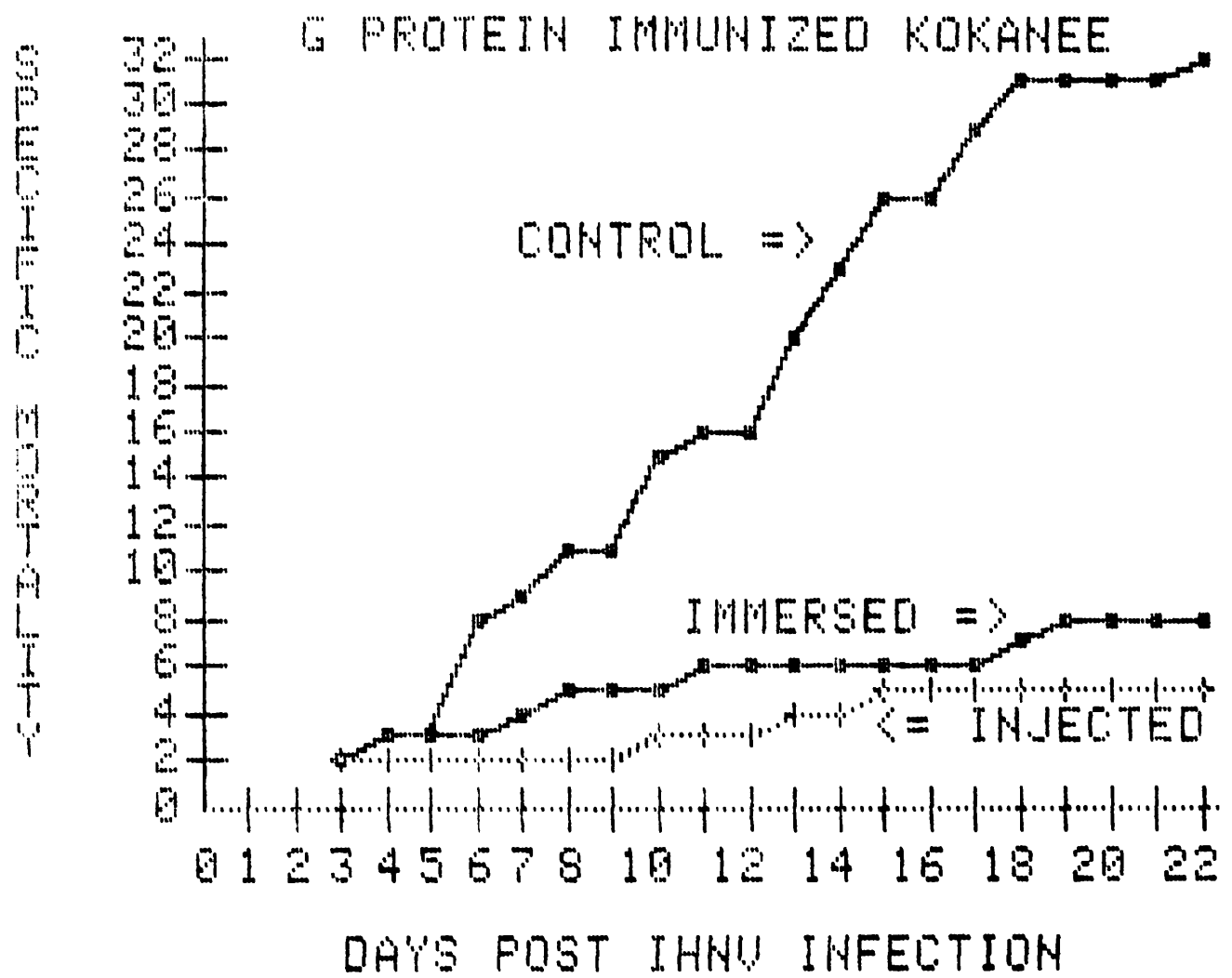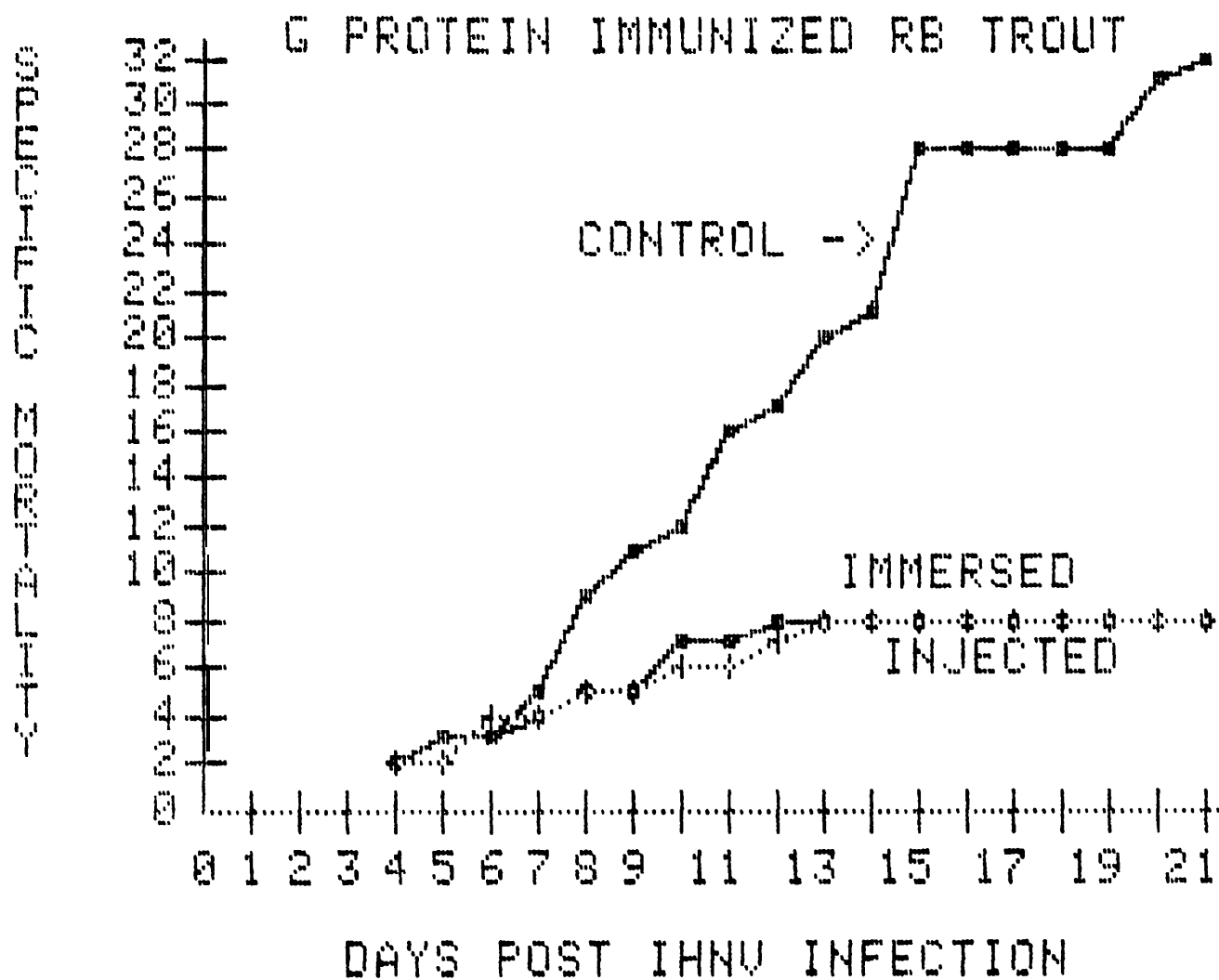
Figure 7

Figure 8

Figure 9

Table 1. <u>Effect of Triton X-100 Concentration on Fish.</u> Fish were
exposed to varying percentages of Triton X-100 in water for the
times indicated.   The numbers shown in each column represent the
number of fish dying from the effects of the detergent in water in
per cent mortality.   There were ten fish in each sample group.

		TIME		
PER CENT TRITON X-100	1 MIN	2 MIN	5 MIN	15 HOURS
0.025	100	—		—
0.020	90	100	—	—
0.015	10	10	10	10
0.010	0	0	10	10
0.005	0	0	0	0

Table 2. LD50 Determination for Vaccinated and Control Fish.
The LD50 was determined for each group fo vaccinated and control
fish. The data are presented in Figures 2-6 and Table 3.

			LD50*		
EXP	FISH SPECIES	STOCK LOCATION	CONTROLS	IMMERSED	INOCULATED
1	Kokanee	Oak Springs	1.46×10^4	____	____
2	Rainbow	Roaring River	0.09×10^3	____	____
3	Kokanee	Wizard Falls	1.47×10^3	3.89×10^3	____
4	Kokanee	Wizard Falls	5.26×10^4	2.51×10^5	4.9×10^5
5	Kokanee	Wizard Falls	2.28×10^3	2.81×10^3	5.0×10^4

*LD50 expressed as TCID 50/ml

Table 3. Comparison of immersion and inoculation vaccination for IHNV glycoprotein in salmon and trout fry.
The fish were challenged by bath with virulent IHNV at the dilutions indicated at 30-35 days after vaccination. The fish were vaccinated with purified glycoprotein from the Round Butte strain of IHNV.

VIRUS DILUTION[a]	INOCULATED				IMMERSED				CONTROL		
	No.	SL	%	RPS	No.	SL	%	RPS	No.	SL	%
Exp.1 -2	25	2	8	89	25	1	4	94	25	18	72
-3	25	0	0	100	25	4	16	71	25	14	56
-4	25	0	0	100	25	0	0	–	25	3	12
-5	25	3	12	–	25	0	0	–	25	0	0
Exp.2 -2	25	4	16	78	25	4	16	78	25	18	72
-3	25	3	12	77	25	4	16	69	25	13	52
-4	25	1	4	0	25	0	0	100	25	1	4
-5	25	0	0	–	25	0	0	100	25	0	0
Exp.3 -2	25	8	32	62	25	17	68	19	25	21	84
-3	25	3	12	80	25	6	24	60	25	15	60
-4	25	0	0	100	25	1	4	92	25	12	48
-5	25	0	0	100	25	0	0	100	25	8	32
Exp.4 -2	25	14	56	44	25	18	72	28	25	25	100
-3	25	5	20	80	25	7	24	72	25	25	100
-4	25	0	0	100	25	2	8	86	25	14	56
-5	25	0	0	100	25	0	0	100	25	3	12
Exp.5 -3	24	18	75	25	25	23	92	8	32	32	100
-4	26	13	50	50	25	15	60	40	37	37	100
-5	22	1	4.5	93	25	5	20	70	30	20	67
-6	23	0	0	100	27	3	11	35	30	5	17

No.= Number of fish in group
SL= Specific Loss, i.e. number of fish dying from IHNV infection
% - Per cent mortality
RPS - Relative per cent survival (See Materials and Methods)
[a]Virus dilutions are shown as ten-fold dilutions of a stock of virus (ca. 2 x 10 TCID50/ml).

Table 4. A comparison of immersion and inoculation vaccination for IHNV glycoprotein. (Cross Protection)
Kokanee fry were vaccinated with purified glycoprotein from the Round Butte strain of IHNV and after 30 days challenged with virulent IHNV (Hagerman Valley strain) at the dilutions indicated

VIRUS DILUTION[a]	INOCULATED				IMMERSED				CONTROL		
	No.	SL	%	RPS	No.	SL	%	RPS	No.	SL	%
-2	24	10	42	51	25	8	32	62	20	17	85
-3	12	8	62	13	23	9	39	45	17	12	71
-4	9	1	11	82	5	3	60	0	25	15	60
-5	20	5	25	50	15	6	40	20	24	12	50

No. = Number of fish in group
SL = Specific Loss, i.e. number of fish dying from IHNV infection
% = Per Cent Mortality
RPS = Relative percent survival (See Materials and Methods)
[a]Virus dilutions are shown as ten -fold dilutions of a stock of virus which had a titer of 1×10^{a} TCID50/ml.

AMINO ACID COMPOSITION AND TERMINAL SEQUENCE ANALYSIS

OF THE GLYCOPROTEIN GENE FOR INFECTIOUS HEMATOPOIETIC NECROSIS

VIRUS: IDENTIFICATION OF THE READING FRAME ON THE CDNA SEQUENCE.

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INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV), a rhabdovirus of fish, most closely resembles rabies virus in its virion proteins. The virus contains a polymerase or L protein of 153,000 daltons; a glycoprotein of 67,000 daltons; a nucleocapsid protein, N, of 41,000 daltons, and two matrix proteins or M-l and M-2 of 21,000 and 17,500 daltons respectively (ref.). In addition, IHNV encodes a gene for a nonvirion protein of 12,000 daltons (Kurath and Leong, 1985).

The viral glycoprotein is a membrane-associated molecule which forms spike-like projections on the surface of mature IHN virions and is responsible for the induction and binding of virus-neutralizing antibodies to the virus (ref.) To define the antigenic and immunogenic properties of the IHN virus glycoprotein, we have cloned a cDNA copy of its mRNA sequence into pUC8. Here we describe the characterization of this cloned gene and its deduced amino acid sequence.

MATERIALS AND METHODS

Cells and Virus. The IHNV used in this study was isolated in 1975
from an adult steelhead trout at the Round Butte hatchery in
central Oregon. The virus was propagated in a chinook salmon
embryo cell line (CHSE-214) obtained from J. L. Fryer, Department
of Microbiology, Oregon State University, Corvallis. The virus
was grown and purified as described previously (Kurath and Leong,
1985).

Preparation of viral RNAs. Viral genome RNA was prepared from
purified virus as described previously (Kurath and Leong, 1985).
Intracellular viral messenger RNA was prepared from CHSE-214
cells infected at a multiplicity of 10 and held at 16° C for
24-28 hours when ca. 25% of the cell monolayer exhibited
cytopathic effect. The cell monolayers were first rinsed three
times with ice-cold Tris-buffered saline. A lysing solution
consisting of 0.5% SDS, 250 ug of proteinase K per ml, 0.1 M NaCl,
5 mM EDTA, and 30 mM Tris-hydrochloride (pH 7.4) was added to 4 ml
for each 150 cm^2 flask. The flasks then were incubated for 1 h at
37 O c. Cell lysates were then pooled, and total nucleic acid was
isolated by two extractions with STE-saturated
phenol-chlorofrom-isoamyl alcohol (25:24:1). The aqueous phase
was extracted with chloroform-isoamyl alcohol (24:1), brought to
0.3 M postassium acetate, and precipitated with 2.5 volumes of
ethanol.

The total nucleic acid preparation was treated with DNase by incubation for 1 h at 37° C in the presence of 0.1 mg of proteinase K-treated DNAse (ref.) per ml, 10 mM $CaCl_2$, 10 mM MgCl 20 mM Tris-hydrochloride (pH 7.8), and 2 mM vanadyl ribonucleoside complexes (Bethesda Research Laboratories). Reactions were stopped by the addition of EDTA to 50 mM. RNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) containing 0.1% 8-hydroxyquinoline until the dye remained yellow, indicating the complete removal of the vanadyl ribonucleoside complexes. The aqueous phase then was brought to 0.3 M potassium acetate and precipitated with 2.5 volumes of ethanol. The RNA precipitates were resuspended in water, measured spectrophotometrically for concentration and purity, and stored at -70° C.

Polyadenylated RNA was selected from the total RNA preapration by column chromatography with oligodeoxythymidylic acid-cellulose by a modified procedure of Aviv and Leder ( ). RNA was suspended in 1 mM EDTA and heat denatured at 65° C for 10 min. After cooling the RNA on ice, Tris-hydrochloride (pH 7.5) and NaCl were added to 10 mM and 0.5 M, respectively. Up to 10 mg of RNA was loaded onto a 8-ml column of oligodeoxytheymidylic acid-cellulose and washed extensively with binding buffer (10 mM Tris-hydrochloride [pH 7.5], 0.5 M NaCl). Bound RNA was eluted by washing with 10 mM Tris-hydrochloride (pH 7.5), and fractions containing RNA were pooled, brought to 0.3 M potassium acetate, and precipitated with 2.5 volumes of ethanol. Precipitated RNA was resuspended in diethyl pyrocarbonate-treated water,

quantitiated, and stored at -70° C. Diethyl pyrocarbonate was used to inactivate RNases in solutions and glassware.

Cloning of viral mRNA species. Polyadenylated RNA was isolated from IHNV-infected CHSE-214 cells as described above. For use as 3 cloning template, this RNA was passed twice over an oligodeoxythymidylic acid-cellulose column to remove all detectable host cell ribosomal RNA. The preparation of double-stranded cDNA was carried out by a procedure described by C. Rice (California Institute Technology, Pasadena, California, personal communication) and modified to include 4 mM sodium pyrophosphate in the first-strand cDNA reaction. The pyrophosphate instread of actinomycin D to prevent the formation of the terminal hairpin loop and eliminate the need for S1 nuclease digestion.

Briefly, 4 ug of polyadenylated RNA was reverse transcribed to synthesize 1.05 ug of single-stranded cDNA in a creaction containing the RNA template, an oligothymidylaic acid (12-18) primer, placental RNase inhibitor (Enzo Biochemicals, Inc.), and reverse transcriptase (life Science Division, The Mogul Corp.). The cDNA product was separated from the other reactants in the mixture by phenol extraction and ethanol precipitation in 2.5 M ammonium acetate. The second-strand synthesis was carried out in a reaction mixture containing 100 mM HEPES (pH 6.9); 4 mM $MgCl_2$; 15 mM 2-mercaptoethanol; 70 mM KC1; 0.1 mM each dATP, dCTP, and

dGTP; 50 uM dTTP; 0.15 mM beta Nicotinic Adenine dinucleotide; 5 units of E. coli ligase (New England Biolabs); 0.65 units of RNase H (Bethesda Research Lab., Inc.); 20 units of E. coli DNA polymerase I (New England Biolabs); and 10 uCi alpha-^{32}P-dTTP (2,637 Ci/mmol) at 12 $^{\circ}$ C for 1 h and then at room temperature for another hour. The reaction yielded 2 ug total of double-stranded cDNA. Tails of ca. 20 dCMP residues were added to the products of this reaction with terminal deoxynucleotidyl transferase. The tailed double-stranded cDNA product was analyzed by alkaline agarose gel electrophoresis. Full length copies of all 5 bands of viral mRNA were obtained by this procedure (Figure 1).

The cDNA band for the glycoprotein gene was isolated after preparative gel electrophoresis in low-melting temperature agarose as previously described (Kurath et al., 1985). The plasmid vector, pUC8, was cleaved with the restriction endonculease Pst I (Bethesda Research Laboraotires), and deoxyguanylic acid tails of ca. 15 residues were added. The deoxyguanylic acid-tailed vector and deoxycytidylic acid-tailed cDNA were annealed at a molar ratio of 1:1 (ref.), and this DNA was used to transform the C600 SC181 strain of Escherichia coli K-12 (ref.). Twenty-five transformants which contained specific sequences homologous to a G-specific probe prepared as previously described (Kurath et al., 1985) were found. An analysis of seven of these clones indicated that the recombinant plasmids contained inserts ranging in size from 1175 to 1600 base pairs, the approximate size of the complete glycoprotein mRNA.

Isolation of plasmid DNA, preparation of nick-translated probes, and colony blot hybridizations. The procedures used to isolate plasmid DNA, prepare nick-translated probes, and prepare colony blots have been described previously (Kurath et al., 1985).

Restriction Endonuclease Map. The restriction endonuclease Pst I (Bethesda Research Laboratories) was used to cleave 10 ug of purified plasmid and the released cloned insert DNA was separated from the plasmid vector by electrophoresis on a 10% gel of low melting temperature agarose (Seakem, FMC). The isolated insert DNA was used for all subsequent restriction enzyme analyses as described by Maniatis, Fritsch, and Sambrook, 1982. Accurate determination the DNA fragments after restriction endonuclease digestion was made by polyacrylamde gel electrophoresis as described (Kurath et al., 1985).

DNA sequence analysis. One cDNA clone containing the entire coding sequence of the IHNV glycoprotein mRNA (pG8) was used for all sequence analyses. DNA sequence analysis was by the Sanger chain termination method (Sanger et al., 1977) on DNA segments which had been subcloned in both orientations in the M13 cloning vectors mp18 and mp19 (ref.).

Preparation of cloned plasmids carrying a complete copy of the
G-protein mRNA. A complete copy of the viral glycoprotein or
G-protein mRNA was synthesized from a preparation of
polyadenylated viral mRNA. The double-stranded cDNA copies was
tailed with deoxycytidylic acid with terminal deoxynucleotidyl
transferase and the cDNA product migrating at a position
appropriate for a full-length copy of the G-protein gene was
isolated from an agarose gel before cloning in the plasmid vector
pUC8. At least 4 clones containing cDNA inserts which appeared to
be full-length copies of the G-protein gene (ca. 1600 base pairs)
were identified. One of these clones, pG8, was used for all
subsequent analyses reported here. The other three clones were
identical to pG8 by sequence analysis (data not shown).

Restriction map of pG8. Restriction enzyme analysis of pG8
was performed by multiple restriction enzyme digestions of
purified segments of the cloned insert. The orientation of the
viral gene in the plasmid vector was determined by Southern blot
(ref.) analysis with probes prepared from a plasmid containing
sequences from the 3' end (pG480) of the viral mRNA as described
previously (Kurath et al., 1985). There are three Taq I sites in
the viral gene which when cleaved generates 4 fragments of 590-bp
(Taq A), 515-bp (Taq B), 265-bp (Taq C), and 230-bp (Taq D) (See
Figure 2). The Taq C and D fragments were cloned in both
orientations in the M13 cloning vectors, mp8 and mp9. In

addition, the Act I A fragment was cloned in the M13 cloning vectors, mp18 and mp19.

Partial nucleotide sequence of the IHNV glycoprotein gene.
The DNA sequences of clones generated from the Taq C and D fragments and the Act I A fragment were determined by the dideoxynucleotide termination method (ref.). All of the regions were sequenced two or three times to ensure the correctness of the sequence. In addition three additional clones were analyzed by scanning the sequence with a single ddATP reaction to verify the accuracy of the cloning procedure.

The orientation of the cloned G gene in pG8 was determined by Southern hybridization with a cloned sequence of the G gene from the 5' end of the G mRNA which has been described (Kurath and Leong, 1985). The G gene is inserted into pUC8 with the cDNA sequence from the 5'end of the G mRNA , i.e. the N terminus of the virual gene, nearest the leftward end of the multiple cloning site and the lac Z gene promoter (Figure 3). Subsequent DNA sequence analysis verified this conclusion by indicating a string of 17 G residues in the coding strand taken from the Taq C fragment cloned in Mp18. The synthesis of the cDNA for the G mRNA adds C residues to the 3' end of the noncoding cDNA strand and adds a poly C tails to a string of A residues at the 3' end of the coding strand for each mRNA. Likewise, a string of G residues would only be found at the 5' end of the coding strand for the G mRNA (Figure 4 and 6).

<u>Correlation of amino acid sequence data.</u>

An analysis of the possible amino acid sequences encoded by the DNA sequence derived from the 5' end of the G gene mRNA is shown in Figure 5. Three possible reading frames are indicated. However, there are termination sequences in Reading Frame 3 and therefore, only Reading Frames 1 and 2 have been considered for further analysis of the gene.

DISCUSSION

The complete sequence of the mRNA encoding the G protein for IHNV has been cloned into the vector, pUC8. The entire sequence beginning with the initation codon ATG (residues 8 to 10) is approximately 1537 residues in length. The predicted protein sequence is 512 amino acids (estimated molecular weight 51,200 without carbohydrate) and is in agreement with the nonglycosylated G protein produced by IHNV in the presence of tunicamyin (Hsu and Leong, data not shown).

It is difficult at this time to compare the DNA sequence and derived amino acid sequence of the IHNV G protein with other known rhabdoviruses. The data that is available is too preliminary to make such studies now.

LEGENDS

Figure 1. Electrophoresis of double stranded cDNA prepared for insertion into pUC8. The double-stranded cDNA was tailed with poly dC as described in Materials and Methods and then analyzed by gel electrophoresis in 1% agarose in tris-acetate buffer. The arros indicate from high to low molecular weight species the cDNA for L, G, N, M1-M2, and NV. This gel indicates that complete cDNA copies to all mRNA species were synthesized in these reactions. Size markers of lambda DNA cleaved with HindIII were also included as indicated.

Figure 2. Restriction map of the glycoprotein gene insert in pG8. Sites for restriction enzymes used in sequencing are shown. The left end corresponds to the 5' end of the G mRNA. The letters correspond to restriction endonucleases as follows: P, Pst I; A, Ava I; E, Eco R1; B, Bgl II; T, Taq I; X, Xba I; Acc, Acc I; and C, Cla I. The Taq C and D fragments are on the left and right ends of the insert respectively.

Figure 3. Orientation of the G gene in pG8.

Figure 4. Nucleotide sequence of the 5'end of the G mRNA insert in pG8.

Figure 5. Nucleotide and predicted amino acid sequence of the IHNV G mRNA and G protein.

| Lamda DNA | IHNV cDNA | Lambda DNA |

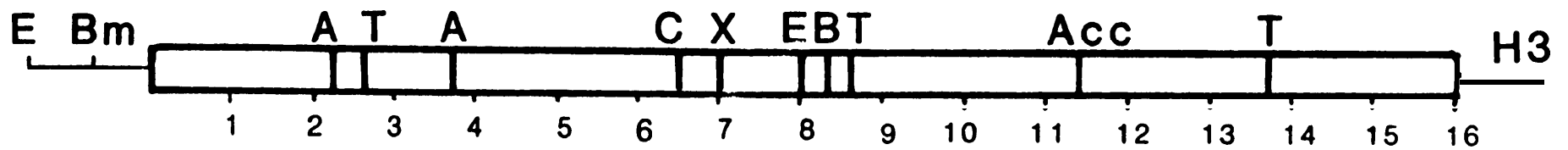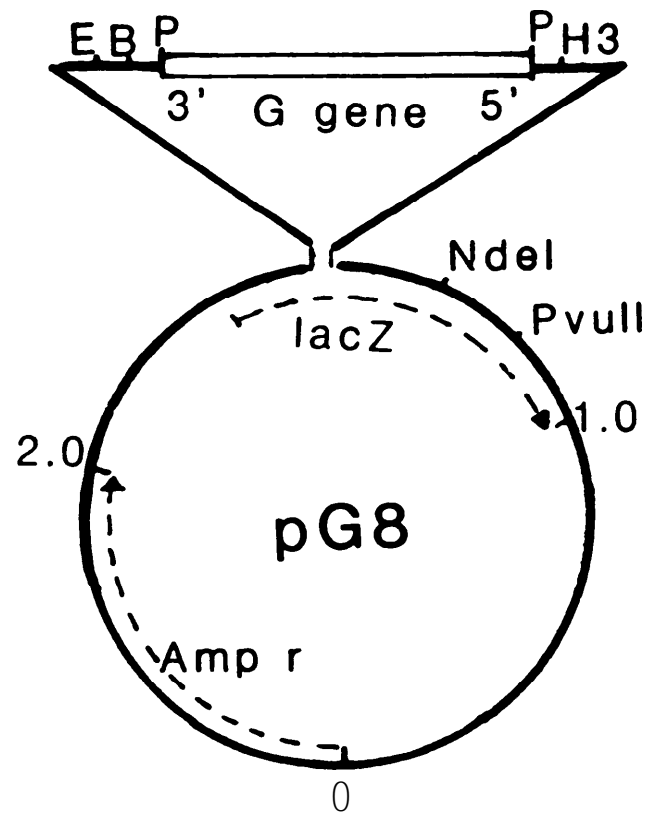Figure 1

MAP OF IHNV GLYCOPROTEIN GENE

Figure 2

Figure 3

Figure 6. <u>Strategy for Synthesizing cDNA copies of IHNV mRNA species.</u>